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REVIEW ARTICLE

Transgenic woody plants for biofuel

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Abstract: Transgenic trees as a new source for biofuel have brought a great interest in tree biotechnology. Genetically modifying forest trees for ethanol production have advantages in technical challenges, costs, environmental concerns, and financial problems over some of crops. Genetic engineering of forest trees can be used to reduce the level of lignin, to produce the fast-growing trees, to develop trees with higher cellulose, and to allow the trees to be grown more widely. Trees can establish themselves in the field with less care of farmers, compared to most of crops. Transgenic crops as a new source for biofuel have been recently reviewed in several reviews. Here, we overview transgenic woody plants as a new source for biofuel including genetically modified woody plants and environment; main focus of woody plants genetic modifications; solar to chemical energy transfer; cellulose biosynthesis; lignin biosynthesis; and cellulosic ethanol as biofuel.

Keywords: biofuel, cell wall, cellulose, lignin, transgenic tree

Introduction

Transgenic trees can be a new energy source of biofuel like ethanol (Chen et al. 2013; Cho et al. 2011; Demain et al. 2005). Genetic engineering approach has been used to change the property of the wood, to increase the amount of cellulose, to reduce the amount of lignin (Batard et al. 1997; Baucher et al. 1996; Bell-Lelong et al. 1997; Busam et al. 1997; Byrt et al. 2012). Lignin interferes with efforts to turn cellulose into biofuel like ethanol. Because lignin provides trees with structural stiffness and resistance to pests, reducing lignin too much could lead to wobbly, vulnerable trees (Landry et al. 1995; Lee et al. 1997; Lorenzen et al. 1996). Therefore, production of transgenic tree with a certain amount of lignin and normal growth and development will be more attractive for biofuel energy industry (Demain et al. 2005; Meyer et al. 1996; Mizutani et al. 1997). Although, these projects will face resistance from others who see trees as majestic symbols of pristine nature that should not be genetically altered like corn and soybeans.

Ethanol is mainly made from the starch in grain. To increase the supply in the energy industry, scientists are looking at using cellulose, a main component of wood in trees, to produce ethanol. Trees are good sources of cellulose and are also good at absorbing carbon dioxide in helping to fight global warming (Demain et al. 2005). Trees can be cut as needed rather than having to be harvested at a given time each year like a crop. However, the cellulose is covered by lignin, which makes it difficult for enzymes to reach the cellulose and to break it down into simple sugars that can be converted to ethanol (Ruegger et al. 1999; Sewalt et al. 1997; Vignols et al. 1995). Pulp and paper companies break down lignin using acids and steam. Ethanol producers would have to do the same. Reducing these steps could save at least 10 cents a gallon in ethanol costs. Genetic engineering plays an important role in modifying trees to produce transgenic trees with a certain amount of lignin and with normal growth (Busam et al. 1997; Chapple et al. 1992; Hawkins et al. 1997; Inoue et al. 1998; Kajita et al. 1997; Kajita et al. 1996; Koopmann et al. 1999).

Transgenic trees as a new source of biofuel bring promising in the field of tree biotechnology and forest and energy industries (Demain et al. 2005). Several companies are being active in pursuing genetic engineering of forest trees, such as developing a low-lignin eucalyptus that it hopes to sell in South America, where the fast-growing trees are already used for pulp and paper, developing a eucalyptus genetically engineered to survive cold snaps, generating transgenic trees with salt and cold tolerance, and producing the trees to be grown more widely (Klinke et al. 2004; Madore and Grodzinski 1984; Mosier et al. 2005; Ohsugi

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and Huber 1987). Transgenic trees provide great potential for biofuel (Demain et al. 2005; Klinke et al. 2004; Turgeon and Wimmers 1988). Here, we overview transgenic woody plants as a new source for biofuel including (1) genetically modified tree and environment; (2) main focus of tree genetic modifications; (3) solar to chemical energy transfer; (4) cellulose biosynthesis; (5) lignin biosynthesis; and (6) cellulosic ethanol as biofuel.

Genetically modified tree and environment

There is growing pressure to commercialize the numerous genetically modified tree species that have been modified with a variety of transgenes for plantations and for biofuel worldwide (Demain et al. 2005; Noctor and Foyer 1998; Tang 2003; Tang and Newton 2005). It has been reported that genetically modified crops save carbon emissions by reducing pesticide use through insecticidal *B.t.* crops and by sequestering carbon in the soil through conservation tillage with herbicide tolerant crops (Ohsugi and Huber 1987; Tuominen et al. 1995; Turgeon and Wimmers 1988). In 2005, it claims, the combined savings were equivalent to 9 million metric tons of carbon dioxide, or removing 4 million cars from the road. And looking to the future, even greater contributions could be made through cultivation of additional areas of genetically modified energy crops to produce ethanol and biodiesel (Noctor and Foyer 1998; Tang and Newton 2004; Tang et al. 2006).

Genetically modified tree species include tropical trees such as banana, avocado, grapefruit, lime, papaya and coffee, horticultural fruits such as apple, plum, pear and walnut, and forest and shade trees such as eucalyptus, American chestnut, American elm, poplar, cottonwood, aspen, spruce, and pine (Noctor and Foyer 1998; Robinson 1999; Tuominen et al. 1995). Transgenic traits range from disease or insect resistance and herbicide tolerance, to lignin modifications, sterility, and bioremediation in Belgium, Canada, France, Finland, New Zealand, Norway, Portugal, Spain and Sweden, Brazil, China, Chile, South Africa and Uruguay (Meyer et al. 1996; Noctor and Foyer 1998; Robinson 1999). Four traits accounted for 80 percent of the permit applications: herbicide tolerance, marker genes, insect resistance, and lignin modification. Of the tree species involved, *Populus*, *Pinus*, *Liquidambar*, and *Eucalyptus* account for 85 percent of applications. However, genetically modified trees have some of the potential hazards of genetically modified crops and genetically modified organisms. Table 1 lists some of the potential hazards of transgenic trees, which shared with transgenic crops (Tuominen et al. 1995; Weigel and Nilsson 1995; Wingate et al. 1988).

Table 1: Influence of genetically modified tree species on environment

	Potential hazards of genetically modified tree species	References
1	Synthetic genes and gene products new to evolution could be toxic for humans and other animals or provoke serious immune reactions and increase the risk of human health if they are not treated well and wisely.	(Tuominen, et al. 1995; Weigel and Nilsson 1995; Wingate, et al. 1988)
2	The uncontrollable, imprecise process involved in making transgenic species can generate unintended toxic and immunogenic products, exacerbated by the instability of the transgenic lines	(Ohsugi and Huber 1987; Wingate, et al. 1988)
3	There is an opportunity that endogenous viruses, in which cause diseases, could be activated by the transgenic process some times.	(Bell-Lelong, et al. 1997; Landry, et al. 1995; Lee, et al. 1997; Lorenzen, et al. 1996)
4	The synthetic genes in transgenic plants, including copies of genes from bacteria and viruses that cause disease as well as antibiotic resistance genes, may be transferred to other species via pollen, or by direct integration into other genomes in horizontal gene transfer	(Baucher, et al. 1996; Wingate, et al. 1988)
5	Disease-causing viruses and bacteria are created by horizontal transfer and recombination of the synthetic genes and genetic modification is nothing if not facilitated and greatly enhanced horizontal gene transfer and recombination	(Joyce and Stewart 2012; Jung, et al. 2012; Kajita, et al. 1997)
6	Transgenic plants DNA are designed to invade genomes and insertion into the genome of animals including human beings results in insertion mutagenesis some of which may trigger cancer	(Vignols, et al. 1995; Warren 1996; Weigel and Nilsson 1995)
7	Herbicide tolerant transgenic plants accumulate herbicide and herbicide residues that could be highly toxic to humans and animals as well as plants	(Weigel and Nilsson 1995; Wingate, et al. 1988)

It has been claimed that conifer pollen dispersed to between 6 and 800 m from a source (Dharmawardhana et al. 1999; Tang et al. 2004; Tang and Newton 2005; Whetten and Sederoff 1995). Eucalyptus pollen is spread by small insects, which can carry pollen to distances of 1.6 km. It is essentially impossible to contain transgenic trees. The probability that pine seeds are transported further than one km from a source was nearly 100 percent (Dharmawardhana et al. 1999; Tang et al. 2004; Tang and Newton 2005; Whetten and Sederoff 1995). Regulators are now suggesting that regulations should be made to accommodate the uncontrolled release of transgenic trees with transgenes for herbicide tolerance, insect resistance or low lignin content (Rasmussen and Dixon 1999; Samuels et al. 2002; Zhong et al. 1998). A recent field study showed that the trees with reduced lignin decomposed more rapidly in the soil and that decay was associated with major restructuring of the soil microbial communities, the adverse impacts of which have yet to be fully evaluated (Tang and Newton 2003; Zhong et al. 1998).

There has been a suggestion of using old forests as buffer zones to contain transgenic trees (Tang and Newton 2003; Zhong et al. 1998). However, scientists in the United States see transgenic contamination as inevitable, and introducing transgenic forest trees as opening a Pandora's box in ecological term. It could be a recipe for disaster as transgenic tree pollen contaminates indigenous species in the old forest and undermine its tightly balanced circular ecology that's vital for regulating climate (Tuominen et al. 1995; Zhong et al. 1998). Viral gene vectors have also been developed to rapidly produce large quantities of pharmaceutical proteins in plants. Gene silencing provides a means of regulating metabolic pathways and controlling plant diseases, and small synthetic RNA molecules have been developed to control plant viruses (Tuominen et al. 1995; Zhong et al. 1998). Such synthetic RNA molecules are readily delivered using viral vectors, which could be sprayed onto forest stands from helicopters. Small RNA molecules require careful and extensive safety evaluations (Saathoff et al. 2013; Tuominen et al. 1995; Zhong et al. 1998). Forests sprayed with small RNA vectors will probably have disastrous effects on bystander plants and animals including humans. However, transgenic trees used for the production of biofuel would have much less hazards as concerned. Almost all of transgenic trees are approved to be economically important.

Main focuses of tree genetic modifications

The main focus of genetic modifications in forest trees has been on herbicide tolerance, insect resistance, and flowering control (Koopmann et al. 1999; Meyer et al. 1996), but some other new developments are being made. There is great potential for transgenic trees as a new energy source of biofuel like ethanol. Transgenic poplar with enhanced growth was constructed using a maize uridinediphosphoglycosyltransferase gene accompanied by an *Arabidopsis* gene for acyl-CoA-binding protein, which enhanced the production of the growth hormone indoleacetic acid. The transgenic poplar grew much faster than the unmodified poplar (Noctor and Foyer 1998; Weigel and Nilsson 1995). An ethanol-inducible promoter from the fungus *Aspergillus* driving a GUS color marker gene was used to transform aspen. Ethanol or ethanol vapour, at concentrations as low as 0.5 percent, induced the expression of marker gene (Busam et al. 1997; Mizutani et al. 1997). A bacterial gene for producing mannitol from fructose was used to induce salt- tolerance in Chinese white poplar (*Populus tomentosa*) (Busam et al. 1997; Mizutani et al. 1997; Noctor and Foyer 1998).

Transformation of a poplar hybrid with the tryptophan decarboxylase gene from *Camptotheca acuminate* caused the gene to over-express. The tryptophan decarboxylase converts tryptophan into tryptamine, which provides resistance to caterpillars of *Malacosoma disstria* (Davin and Lewis 2000; Tang et al. 2005; Zhang et al. 2013; Zhong et al. 1998). Excess of tryptamine may result in hallucinogenic tryptamines, but that aspect was not explored. A transcription factor from *Capsicum annuum* (pepper) transferred to pine trees resulted in enhanced multiple stress tolerance such as drought, salt and freezing tolerances. The transcription factor increases polyamine biosynthesis (Robinson 1999; Tuominen et al. 1995), which increases the ability of transgenic plants to tolerance to oxidation stress. The main focus of tree genetic modifications is listed in Table 2.

It seems likely that marker assisted selection may provide the most long lasting and best fruit-tree improvement. Even though most of the work on transgenic forest and fruit trees is well meant and promises rich financial reward, Transgenic trees considered to be commercialized or released should be fully evaluated first (Tang and Newton 2003; Ye 1997; Zhong et al. 1998). A moratorium on release of all transgenic trees is essential because the inevitable spread of transgenes in pollen and seed cannot be prevented (Tang and Newton 2003; Ye 1997; Zhong et al. 1998). Transgenic trees as a source of biofuel will bring great promising to energy industry, but we must be fully evaluated them before the commercialization.

Conversion of light energy into chemical energy

The ability to use solar power to generate liquid carbon-based biofuel has the great potential to generate energy for transportation. Biotechnologists have shown that it is applicable to produce biofuel use biomass derived from solar energy (Davin and Lewis 2000; Everard et al. 1994; Giaquinta et al. 1983; Gibson et al. 2011). Although, researchers are working on direct conversion of solar energy to chemical energy and they demonstrated that light absorbed and converted into electricity by a silicon electrode can help drive a reaction that converts carbon dioxide into carbon monoxide and oxygen. Carbon monoxide is a key ingredient in a process for making synthetic fuels, including methanol, and gasoline (Demain et al. 2005; Everard et al. 1994). Hydrogens can be attached to the carbon and hooked up into long enough chains and the hydrocarbons become liquid at room temperature, which can be used as fuel for a gas tank and cruise (Demain et al. 2005; Noctor and Foyer 1998; Warren 1996). Although it is possible that solar power could run chemical processes to produce liquid fuels, it is more promising to produce biofuel from biomass. Transgenic trees provide more potential as a source of biofuel because trees synthesize sugar with photosynthesis.

The dark reaction takes place in the stroma within the chloroplast, and converts CO_2 to sugar. This reaction doesn't directly need light in order to occur, but it does need the products of the light reaction (ATP and another chemical called NADPH). The dark reaction involves a cycle called the Calvin cycle in which CO_2 and energy from ATP are used to form sugar (Giaquinta et al. 1983; Warren 1996). Actually, notice that the first product of photosynthesis is a three-carbon compound called glyceraldehyde 3-phosphate. Almost immediately, two of these join to form a glucose molecule (Fig. 1). Most plants put CO_2 directly into the Calvin cycle. Thus the first stable organic compound formed is the glyceraldehyde 3-phosphate (Noctor and Foyer 1998; Warren 1996). Plants lessen the amount of water that evaporates by keeping their stomates closed during hot, dry weather. Typically the grass in our yards just turns brown and goes dormant. Plants capture CO_2 in a different way: they do an extra step first, before

doing the Calvin cycle. Plants have a special enzyme that can work better, even at very low CO_2 levels, to grab CO_2 and turn it first into oxaloacetate, which contains four carbons (Madore and Grodzinski 1984; Ohsugi and Huber 1987; Turgeon and Wimmers 1988). The CO_2 is then released from the oxaloacetate and put into the Calvin cycle (Fig. 1).

Table 2: The main focus of tree genetic modifications

	The main focus of tree genetic modifications	References
1	Insect resistance: Poplars and pine modified with the Bt Cry1Ac gene or with a Cry1Ac gene fusion with the cowpea protease inhibitor gene have been extensively deployed.	Strauss et al. 1995
2	Flowering control: Transgenic poplars have been developed.	Strauss et al. 1995
3	Herbicide tolerance: Transgenic poplars and pine have been developed with enhanced growth.	(Tang and Newton 2003), Strauss et al. 1995
4	Inducible expression: An ethanol-inducible promoter from the fungus *Aspergillus* driving a GUS colour marker gene was used to transform aspen. Ethanol or ethanol v(Lee, et al. 1997; Robinson 1999)apour at concentrations as low as 0.5 percent induced the marker gene.	(Tang and Newton 2003), Strauss et al. 1995
5	Salt tolerance: A bacterial gene for producing mannitol from fructose was used to induce salt- tolerance in Chinese white poplar (*Populus tomentosa*).	(Tang and Newton 2003)
6	Resistance to caterpillars: Transformation of a poplar hybrid with the tryptophan decarboxylase gene from *Camptotheca acuminate* caused the gene to over-express. The tryptophan decarboxylase converts tryptophan into tryptamine, which provides resistance to caterpillars of *Malacosoma disstria*.	(Tang and Newton 2003), Strauss et al. 1995]
7	Drought tolerance: A transcription factor from *Capsicum annuum* (pepper) transferred to pine trees resulted in enhanced multiple stress tolerance (drought, salt and freezing). The transcription factor increases polyamine biosynthesis.	(Tang, et al. 2005; Zhong, et al. 1998)
8	Virus resistance: Fruit trees are much targeted by genetic engineers. Papaya and plum trees resistant to virus were the first trees approved, or petitioned for commercial release in the United States, with flagrant disregard of safety	
10	Herbicide tolerance: A grape stilbene synthase gene accompanied by a bar gene for herbicide tolerance was used to transform apple to enhance picied (reveratrol glucoside) production in the apple. Picied is both a phytoelexin for pest control and a health-promoting antioxidant	(Tuominen, et al. 1995)
11	Fungal resistance: Bacterial fire blight disease is a significant problem in pear and apple. Pears were transformed with a gene from a bacteria phage that dissolves the extracellular polysaccharide of the bacterial pest. The transgenic pears were only partially resistant to the bacterial pathogen but researchers thought improvements in the process might be possible	Strauss et al. 1995
12	DNA degradation: Transgenic orange trees with a GUS marker gene driven by a CaMV promoter accompanied by a neomycin antibiotic resistance gene bore fruit that was harvested. The fruit was processed to make juice, to which was added bacterial plasmid DNA, yeast DNA and additional transgenic orange DNA. The orange juice-DNA soup was then pasteurized and stored. The pasteurization and acidic environment of the orange juice degraded all of the added and endogenous DNA molecules to molecular sizes smaller than the size required for bacterial transformation	Strauss et al. 1995, (Wingate, et al. 1988).
13	Frost tolerance: Trifoliate orange (*Poncirus trifoliate*) is a member of the family Rutaceae closely related to Citrus, and sometimes included in that genus, being sufficiently closely related for it to be used as a rootstock for Citrus. The plant is fairly hardy and will tolerate moderate frost and snow, making a large shrub or small tree 4-8 m tall. Because of the relative hardiness of *Poncirus* , citrus grafted onto it are usually hardier than when grown on their own roots. Reducing the generation time can greatly facilitate genetic improvement of the rootstock for commercial citrus production, subject to satisfactory safety assessment.	Strauss et al. 1995, (Davin and Lewis 2000; Everard, et al. 1994)

Photosynthesis is the conversion of light energy into chemical energy by living organisms. It is affected by its surroundings and the rate of photosynthesis is affected by the concentration of carbon dioxide, the intensity of light, and the temperature. Photosynthesis occurs in two stages. In the first phase, in the light reactions, one molecule of the pigment chlorophyll absorbs one photon and loses one electron. This electron is passed to a modified form of chlorophyll called pheophytin, which passes the electron to a quinone molecule, allowing the start of a flow of electrons down an electron transport chain that leads to the ultimate reduction of NADP into NADPH. In addition, it serves to create a proton gradient across the chloroplast membrane; its dissipation is used by ATP synthase for the concomitant synthesis of ATP. The chlorophyll molecule regains the lost electron by taking one from a water molecule through a process called photolysis that releases oxygen gas. During the second phase, the light-independent reactions use the enzyme RUBISCO captures carbon dioxide from the atmosphere and in a process that requires the newly-formed NADPH, called the Calvin-Benson cycle releases three-carbon sugars, which are later combined to

form sucrose and starch.

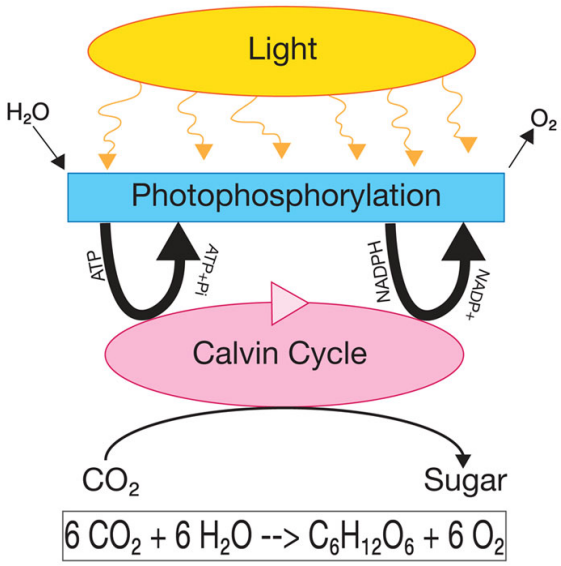

Fig. 1: Photosynthesis and energy conversion

To be more specific, carbon fixation produces an intermediate product, which is then converted to the final carbohydrate products. The carbon skeletons produced by photosynthesis are then variously used to form other organic compounds, such as the building material cellulose, as precursors for lipid and amino acid biosynthesis, or as a fuel in cellular respiration (Davin and Lewis 2000; Everard et al. 1994; Vignols et al. 1995; Warren 1996). Although all cells in the green parts of a plant have chloroplasts, most of the energy is captured in the leaves. The cells in the interior tissues of a leaf, called the mesophyll, can contain between 450,000 and 800,000 chloroplasts for every square millimeter of leaf. The surface of the leaf is uniformly coated with a water-resistant waxy cuticle that protects the leaf from excessive evaporation of water and decreases the absorption of ultraviolet or blue light to reduce heating (Everard et al. 1994; Giaquinta, et al. 1983; Warren 1996). The transparent epidermis layer allows light to pass through to the palisade mesophyll cells where most of the photosynthesis takes place. Plants convert light into chemical energy with a maximum photosynthetic efficiency of approximately 6%. By comparison solar panels convert light into electric energy at a photosynthetic efficiency of approximately 10-20%. Actual plant's photosynthetic efficiency varies with the frequency of the light being converted, light intensity, temperature and proportion of CO_2 in atmosphere (Everard et al. 1994; Giaquinta, et al. 1983; Warren 1996). Sugar produced through photosynthesis is the source of biosynthesis of starch, cellulose, and lignin. Cellulose is the new source of biofuel.

Cellulose biosynthesis

Cellulose synthesis (Fig. 2) is important in plant biochemistry. The basic synthetic event of cellulose synthesis is the polymerization of glucose residues from a substrate such as UDP-glucose to form the homopolymer p-l,4-β-glucan (Hawkins, et al. 1997; Meyer, et al. 1996). The stereochemistry, which is related to the forms and patterns in which cellulose is deposited in nature, imposed by the β-1,4-glycosidic linkage creates a linear, extended glucan chain in which every other glucose residue is rotated -180° with respect to its neighbor (Fig. 2). Cellulose in nature never occurs as a single chain but exists from the time of synthesis as a composite of many chains, called microfibrils (Fig. 2). Chain length can vary among organisms, ranging from a low of -2000 up to -20,000 glucose residues, and virtually nothing is known about how chain length is determined (Dixon, et al. 2001; Samuels, et al. 2002; Ye 1997).

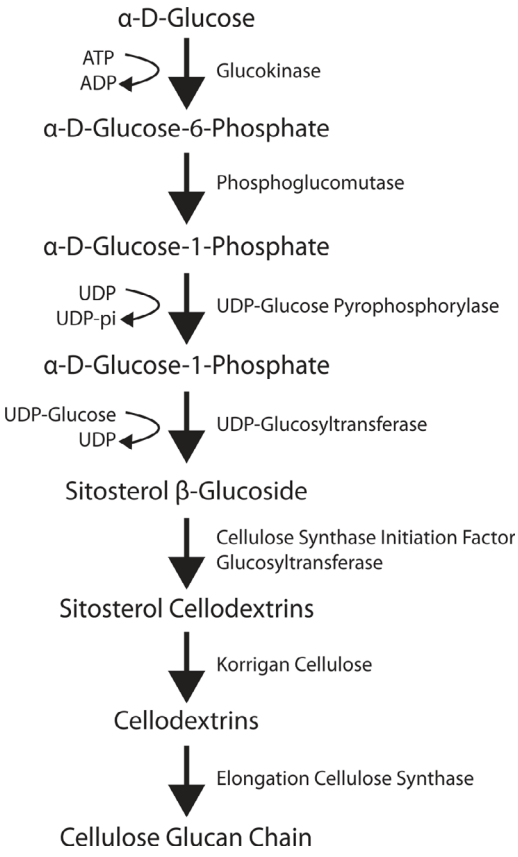

Fig. 2: Cellulose biosynthesis

Cellulose synthesis starts from α-D-Glucose. Glucokinase utilizes water soluble α-D-glucose and one phosphate molecule of an ATP molecule to produce α-D-glucose-6-phosphate, which is converted by phosphoglucomutase to α-D-glucose-1-phosphate. UDP-glucose pyrophosphorylase (UDP-PP) removes one organic phosphate from α-D-glucose-1-phosphate to produce UDP-glucose, which is soluble in the cytoplasm and is the precursor for the generation of microcrystalline cellulose. With polymerization of UDP glucose and formation of glucan chains, the glucan chains then extrude into the plant cell wall where they coalesce to form microfibrils. In microfibrils, the multiple hydroxyl group of the glucose residues of one glucan chain form hydrogen bonds with oxygen molecules of another glucan chain, resulting in firm side-by-side chains of glucans with high tensile strength cellulose microfibrils.

Cellulose provides strength to the primary walls of dividing and elongating cells. In most primary walls, cellulose exists as elementary fibrils that form a complex with xyloglucan (Warren 1996; Whetten et al. 1998). In mature plants, the overall strength of the plant derives mainly from the very thick secondary cell walls characteristic of many mature cell types of the plant. The process of cellulose deposition plays another critical role in determining the development of plant development (Demain et al. 2005; Lorenzen et al. 1996). The process of cellulose synthesis not only involves chain polymerization but also includes mechanisms that determine how no single system has emerged as ideal for the study of cellulose biosynthesis (Demain et al. 2005; Warren 1996; Whetten et al. 1998). The microfibrils from each synthetic site merge to form a large ribbon of cellulose and these ribbons and associated cells tangle and form a floating pellicle that allows the nonmotile, strictly aerobic bacteria to grow in the higher oxygen tension at the surface (Joseleau and Ruel 1997; Mudalkar et al. 2013; Nair et al. 2002). A cellulose synthase activity has been demonstrated, and many sophisticated genetic approaches are available that make it very attractive for developmental studies of cellulose biosynthesis (Byrt et al. 2012; Culleton et al. 2013; Demain et al. 2005).

In higher plants, cotton fibers represent an interesting object of study (Demain et al. 2005; Everard et al. 1994). Cellulose from cotton constitutes more than 90% of the dry weight of the mature fiber cell. Cotton fibers are single cells that elongate from the epidermal layer of the ovule, and they elongate synchronously within the boll. At the transition to secondary wall synthesis, the fibers transiently synthesize callose, followed by massive deposition of secondary wall cellulose (Everard et al. 1994; Noctor and Foyer 1998; Warren 1996), in which the microfibrils are deposited in helical arrays in successive layers of alternating pitch. Functional genomics and mutant studies have played important parts in the identification of genes that are involved in both cellulose and hemicellulose biosynthesis (Fig. 2). Although cellulose biosynthesis has been studied for decades, most steps in this pathway are not yet well understood (Everard et al. 1994; Noctor and Foyer 1998; Warren 1996). Future studies will be geared towards an improved understanding of the biosynthesis of these plant cell-wall polysaccharides, and towards their genetic manipulation to increase polysaccharides for improved cellulosic biofuel production. Recent large grants for biofuel research are aimed at these issues (Turgeon and Wimmers 1988; Vignols et al. 1995).

Increased overall biomass could also be achieved by genetically modifying plants. This could include modification of plant growth regulators. For example, transgenic hybrid poplar with increased gibberelin biosynthesis displayed improved growth and an increase in biomass (Everard et al. 1994; Giaquinta et al. 1983), probably owing to the effects of gibberelin on plant height. There are also several other potential routes to increasing overall plant biomass (Everard et al. 1994; Giaquinta et al. 1983; Ryser et al. 1997). Assuming there are no limitations to the supplies of water, fertilizer or sunlight, feedstock biomass is the product of the solar radiation over the cropping duration. Both increasing biomass and improving cellulose content make transgenic trees more promising as a source of biofuel (Everard et al. 1994; Giaquinta et al. 1983).

Lignin biosynthesis

Lignin is a complex chemical compound most commonly derived from wood and an integral part of the cell walls of plants. Lignin fills the spaces in the cell wall between cellulose, hemicellulose, and pectin components, especially in tracheids, sclereids and xylem (Whetten and Sederoff 1995; Whetten et al. 1998; Wingate et al. 1988). Lignin plays a significant role in the carbon cycle, sequestering atmospheric carbon into the living tissues of woody perennial vegetation. There are three monolignol monomers, methoxylated to various degrees: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 3). The biosynthesis of the monolignols starts with the deamination of phenylalanineand involves successive hydroxylation reactions of the aromatic ring, followed byphenolic *O*-methylation and conversion of the side-chain carboxyl to an alcohol group. Monolignol biosynthesis is presented in Figure 3, which includes the enzymatic conversions that have been shown by in vitro experiments (Whetten and Sederoff 1995; Whetten et al. 1998; Wingate et al. 1988). The hydroxylation and methylation reactions occur at the level of the cinnamicacids and that *p*-coumaric, ferulic, and sinapic acid are subsequently converted to the corresponding monolignols by the sequential action of 4-coumarate:CoA ligase (4CL), Cinnamoyl-CoA reductase (CCR), Cinnamyl alcohol dehydrogenase (CAD). However, a number of in vitro enzymatic assays with heterologously produced enzymes, the identification of novel genes implicated in the pathway, and analyses of mutant and transgenic plants modified in monolignol biosynthesis have cast doubt on this route (Whetten et al. 1998; Zhong et al. 1998).

The pathway of monolignol biosynthesis was proposed from the observation that expression of caffeoyl-CoA *O*-methyltransferase (CCoAOMT) coincided with lignin deposition in differentiating tracheary elements in zinnia and other plant species (Guo et al. 2001; Mavandad et al. 1990; Ni et al. 1996; Ye 1997). The monolignol Para-hydroxyphenyl residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, 4-coumarate:CoA ligase , Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases. The monolignol Guaiacyt residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, Cinnamate 3-hydroxylase, O-methyltransferase, 4-coumarate:CoA ligase, Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases. The monolignol Syringyl residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, Cinnamate 3-hydroxylase, O-methyltransferase, Ferulate 5-hydroxylase,4-coumarate:CoA ligase, Caffeoyl-COA 3-O-methyltransferase, Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases (Fig. 3). Feed-

ing experiments with radiolabeled monolignol glucosides showed that hydroxylation and methylationof the aromatic C3 and C5 positions could also occur at the aldehyde or alcohollevel (Franke et al. 2002; Hawkins et al. 1997; Kajita et al. 1997; Ye 1997), indicating the existence of enzymes able to catalyze these conversions. The aromatic C5 position is hydroxylated and methylated preferentially at the cinnamaldehyde or cinnamyl alcohollevel and that the predominant role for Cinnamoyl-CoA reductase is the reduction of feruloyl-CoAto coniferaldehyde (Hawkins et al. 1997; Kajita et al. 1997; Ye 1997).

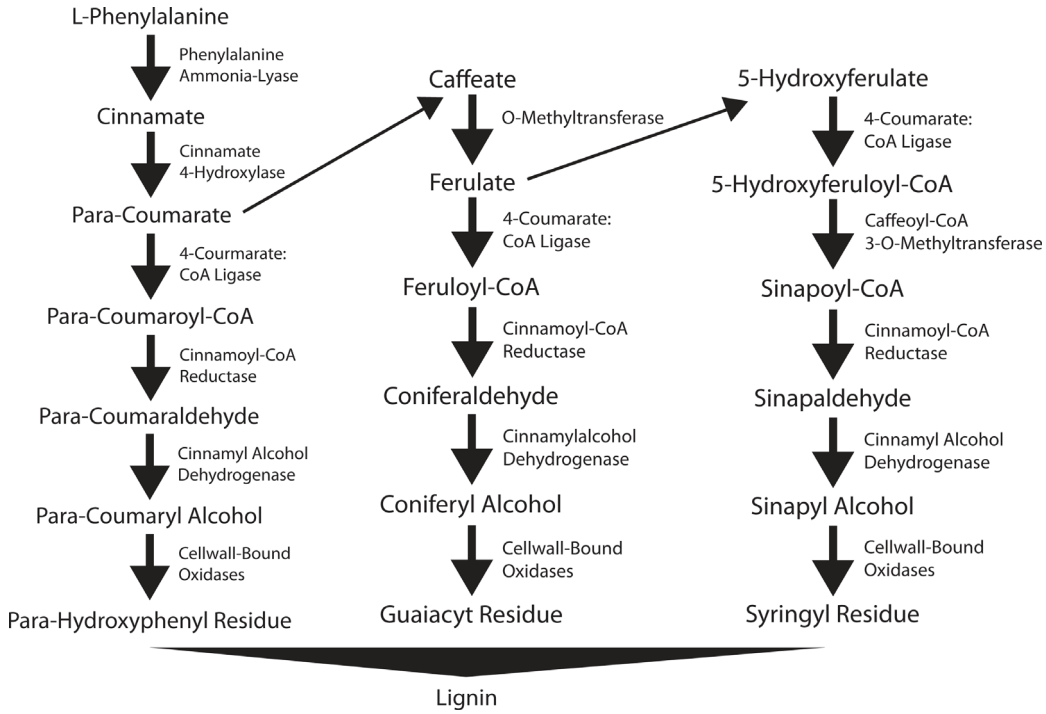

Fig. 3: Monolignol biosynthetic pathways.

Based on lignin compositional analyses, enzymatic assays, and transgenic plants, the production of monolignols is expected to be three major routes. The monolignol Para-hydroxyphenyl residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, 4-coumarate:CoA ligase , Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases. The monolignol Guaiacyt residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, Cinnamate 3-hydroxylase, O-methyltransferase, 4-coumarate:CoA ligase, Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases. The monolignol Syringyl residue is synthesized from L-phenylalanine under the successive reactions of Phenylalanine ammonia-lyase, Cinnamate 4-hydroxylase, Cinnamate 3-hydroxylase, O-methyltransferase, Ferulate 5-hydroxylase,4-coumarate:CoA ligase, Caffeoyl-COA 3-O-methyltransferase, Cinnamoyl-CoA reductase, Cinnamyl alcohol dehydrogenase, Cell wall-bound oxidases.

Cinnamyl alcohol dehydrogenase is a multifunctional enzyme that catalyzes the final reduction of the cinnamaldehydesto the corresponding alcohols. However, a Cinnamyl alcohol dehydrogenase homolog from aspen, sinapyl alcohol dehydrogenase (Giaquinta et al. 1983), which preferentially reduces sinapaldehyde to sinapyl alcohol, was identified. Aspen Cinnamyl alcohol dehydrogenase preferentially reduces coniferaldehyde; therefore, SAD may be the enzyme responsible for the final step in the biosynthesis of sinapyl alcohol (Kajita et al. 1996; Mavandad et al. 1990; Whetten et al. 1998), by incorporating C3H into the scheme for monolignol biosynthesis. In *Arabidopsis*, *p*-coumarate is first converted to *p*coumaroyl-CoA by 4CL, with subsequent conversion to *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate, the substrates for C3H, by *p*-hydroxycinnamoyl-CoA:Dquinate-(CQT) (Guo et al. 2001; Humphreys and Chapple 2002; Kawai et al. 1996). These enzymes, described as reversible enzymes, can convert caffeoyl-shikimate or caffeoyl-quinate into caffeoyl-CoA, the substrate for CCoAOMT (Kajita et al. 1996; Mavandad et al. 1990; Whetten et al. 1998). Although the basic background of lignin biosynthesis is recognized, much more work is needed to establish a model pathway that may be acceptable for most of the plant species.

Cellulosic ethanol as biofuel

Cellulosic ethanol is a type of biofuel produced from lignocellulose, which comprises much of the mass of plants including cellulose, hemicelluloses, and lignin (Demain et al. 2005; Nair et al. 2002). Corn stover, switchgrass, miscanthus, and woodchips are some of the most popular cellulosic materials for ethanol produc-

tion. Cellulosic ethanol is chemically identical to ethanol produced from other sources, such as starch and sugar, but has the advantage that the lignocellulose raw material is highly abundant and diverse (Demain et al. 2005; Nair et al. 2002). However, it differs in that it requires a greater amount of processing to make the sugar monomers available to the microorganisms that are typically used to produce ethanol by fermentation. The first attempt at commercializing a process for ethanol from wood was done in Germany in 1898. It involved the use of dilute acid to hydrolyze the cellulose to glucose, and was able to produce 7.6 L of ethanol per 100 kg of wood waste (Batard et al. 1997; Baucher et al. 1996; Bell-Lelong et al. 1997; Demain et al. 2005). The Germans soon developed an industrial process optimized for yields of around 50 gallons per ton of biomass. This process soon found its way to the United States, culminating in two commercial plants operating in the southeast during World War I. A steady amount of research on dilute acid hydrolysis continued at the USDA's Forest Products Laboratory (Batard et al. 1997; Baucher et al. 1996; Bell-Lelong et al. 1997; Demain et al. 2005; Nair et al. 2002).

In April 2004, Iogen Corporation became the first biotechnology firm business to commercially sell cellulosic ethanol. The primary consumer thus far has been the Canadian government. Currently, the United States government has invested millions of dollars into assisting the commercialization of cellulosic ethanol (Batard et al. 1997; Baucher et al. 1996; Bell-Lelong et al. 1997; Demain et al. 2005; Nair et al. 2002). Another company that appears to be nearing commercialization of cellulosic ethanol is from Spain. Using process and pre-treatment technology, this company is building a 5 million gallon cellulosic ethanol facility in Spain and has recently entered into a strategic research and has created a new and better enzyme mixture which may be used to improve both the efficiencies and cost structure of producing cellulosic ethanol (Batard et al. 1997; Baucher et al. 1996; Bell-Lelong et al. 1997; Demain et al. 2005). In U.S., the first cellulosic ethanol pilot plants have created in Louisiana and expected to achieve mechanical completion of a 1.4 million gallon-per-year, demonstration-scale facility to produce cellulosic ethanol. In addition, the Company's process technology has been licensed and incorporated into a cellulosic ethanol plant in Japan, which is the world's first commercial-scale plant to produce cellulosic ethanol from wood construction waste (Baucher et al. 1996; Demain et al. 2005).

The government of the United States has proposed to expand the use of cellulosic ethanol by announcing a proposed mandate for 35 billion gallons of ethanol by 2017. It is widely recognized that the maximum production of ethanol from corn starch is 15 billion gallons per year, implying a proposed mandate for production of some 20 billion gallons per year of cellulosic ethanol by 2017 (Batard et al. 1997; Baucher et al. 1996; Demain et al. 2005). The government of the United States has a proposed plan including $2 billion funding from 2007-2017 for cellulosic ethanol plants, with an additional $1.6 billion announced by the USDA. In March 2007, the US government awarded $385 million in grants aimed at jumpstarting ethanol production from nontraditional sources like wood chips, switchgrass and citrus peels. Half of the six projects chosen will use thermo-chemical methods and half will use cellulosic ethanol methods (Batard et al. 1997; Baucher et al. 1996; Demain et al. 2005; Klinke et al. 2004; Mosier et al. 2005).

There are two ways of producing alcohol from cellulose. One is the cellulolysis processes which consist of hydrolysis on pretreated lignocellulosic materials followed by fermentation and distillation; another is the gasification that transforms the lignocellulosic raw material into gaseous carbon monoxide and hydrogen (Mosier et al. 2005; Ohsugi and Huber 1987). These gases can be converted to ethanol by fermentation or chemical catalysis. They both include distillation as the final step to isolate the pure ethanol (Table 3).

Table 3: The two ways of producing alcohol from cellulose

Steps	Cellulolysis (biological approach)	Gasification process (thermochemical approach)	References
1	Pretreatment phase to make the lignocellulosic material such as wood or straw amenable to hydrolysis	Complex carbon based molecules are broken apart to access the carbon as carbon monoxide, carbon dioxide and hydrogen are produced	(Demain et al. 2005; Warren 1996)
2	Cellulose hydrolysis (cellulolysis) to break down the molecules into sugars. Separation of the sugar solution from the residual materials of lignin	Convert the carbon monoxide, carbon dioxide and hydrogen into ethanol using the Clostridium ljungdahlii organism	(Demain et al. 2005; Kajita et al. 1996)
3	Microbial fermentation of the sugar solution. Distillation to produce 99.5% pure alcohol.	Ethanol is separated from water by distillation	[(Culleton et al. 2013; Davin and Lewis 2000)

Cellulose is the most abundant material from the resource of plant. To produce biofuel, an effective pretreatment is needed to liberate the cellulose from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step (Everard et al. 1994; Landry et al. 1995). Currently, the available pretreatment techniques include acid hydrolysis, steam explosion, ammonia fiber expansion, alkaline wet oxidation and ozone pretreatment. The cellulose molecules are composed of long chains of sugar molecules of various kinds. In the hydrolysis process, these chains are broken down to free the sugar, before it is fermented for alcohol production (Demain et al. 2005; Klinke et al. 2004). There are two major cellulose hydrolysis

processes: one is chemical reaction using acids, the other is enzymatic reaction using enzyme. Various enzyme companies have contributed significant technological breakthroughs in cellulosic ethanol through the mass production of enzymes for hydrolysis at competitive prices. Some of enzyme companies are developing genetically engineered fungi which would produce large volumes of cellulase, xylanase and hemicellulase enzymes which can be utilized to convert agricultural residues such as corn stover, distiller grains, and woodchips into fermentable sugars which may be used to produce cellulosic ethanol (Demain et al. 2005; Dixon et al. 2001; Everard et al. 1994).

The ability of the fermenting microorganisms to utilize the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol and potentially bio-based chemicals. In recent years, metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress (Demain et al. 2005; Dixon et al. 2001). Microorganisms such as *Zymomonas mobilis* and *Escherichia coli* have been targeted through metabolic engineering for cellulosic ethanol production. Traditionally, yeast (*Saccharomyces cerevisiae*) has been used in brewery industry to produce ethanol from hexoses. Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose is also present in the hydrolysate (Demain et al. 2005; Everard et al. 1994). Yeast cells are especially attractive for cellulosic ethanol processes as they have been used in biotechnology for hundred of years, as they are tolerant to high ethanol and inhibitor concentrations and as they can grow at low pH values which avoids bacterial contaminations (Demain et al. 2005; Mosier et al. 2005).

The gasification process does not rely on chemical decomposition of the cellulose chain. Instead of breaking the cellulose into sugar molecules, the carbon in the raw material is converted into synthesis gas, using what amounts to partial combustion. The carbon monoxide, carbon dioxide and hydrogen may then be fed into a special kind of fermenter. Alternatively, the synthesis gas from gasification may be fed to a catalytic reactor where the synthesis gas is used to produce ethanol and other higher alcohols through a thermochemical process (Demain et al. 2005; Lorenzen et al. 1996). This process can also generate other types of liquid fuels, an alternative concept under investigation by at least one biofuel company (Dixon et al. 2001; Giaquinta et al. 1983). As of 2007, ethanol is produced mostly from sugars or starches, obtained from fruits and grains. In contrast, cellulosic ethanol is obtained from cellulose, the main component of wood, straw and much of the structure of plants. Since cellulose cannot be digested by humans, the production of cellulose does not compete with the production of food, other than conversion of land from food production to cellulose production. Moreover, even land marginal for agriculture could be planted with cellulose-producing crops, resulting in enough production to substitute for all the current oil imports into the United States (Dixon et al. 2001; Giaquinta et al. 1983).

Currently, corn is easier and less expensive to process into ethanol in comparison to cellulosic ethanol. The Department of Energy estimates that it costs about $2.20 per gallon to produce cellulosic ethanol, which is twice as much as ethanol from corn. One of the major reasons for increasing the use of biofuel is to reduce greenhouse gas emissions (Demain et al. 2005; Ohsugi and Huber 1987). In comparison to gasoline, ethanol burns cleaner with a greater efficiency, thus putting less carbon dioxide and overall pollution in the air. Additionally, only low levels of smog are produced from combustion (Giaquinta et al. 1983; Humphreys and Chapple 2002; Joyce and Stewart 2012). According to the U.S. Department of Energy, ethanol from cellulose reduces greenhouse gas emission by 90 percent, when compared to gasoline and in comparison to corn-based ethanol which decreases emissions by 10 to 20 percent (Demain et al. 2005; Warren 1996). Carbon dioxide gas emissions are shown to be 85% lower than those from gasoline. Cellulosic ethanol contributes little to the greenhouse effect and has a five times better net energy balance than corn-based (Demain et al. 2005; Warren 1996). When used as a fuel, cellulosic ethanol releases less sulfur, carbon monoxide, particulates, and greenhouse gases. Cellulosic ethanol should earn producers carbon reduction credits, higher than those given to producers who grow corn for ethanol, which is about 3 to 20 cents per gallon (Giaquinta et al. 1983; Humphreys and Chapple 2002; Joyce and Stewart 2012). Transgenic trees can be a great resource for the production of cellulosic ethanol. There is huge potential to produce biofuel from woodchips derived from transgenic trees after reducing the content of lignin and increasing the content of cellulose through genetic modification. Production of transgenic pine (Fig. 4) provides great potential for transgenic tree as a source for biofuel (Tang and Newton 2005; Tang et al. 2006; Tang et al. 2007).

Transgenic trees can be a great resource for the production of cellulosic ethanol. The entire plant of transgenic trees can be used when producing cellulosic ethanol. Transgenic trees that produce biomass materials require fewer inputs, such as fertilizer, herbicides, and other chemicals that can pose risks to wildlife. Their extensive roots of transgenic trees improve soil quality, reduce erosion, and increase nutrient capture. Transgenic trees provide an environment for diverse wildlife habitation, mainly insects and ground birds. It is also tolerant to poor soils, flooding, and drought; improves soil quality and prevents erosion due its type of root system. Transgenic trees reduce soil erosion, enhance water quality, and increases wildlife habitat. Cellulosic ethanol commercialization based on transgenic trees can contribute to a successful renewable fuels future. Transgenic trees as a new source for biofuel offers significant opportunities for tree farmers, biotechnology firms, project developers, and energy investors.

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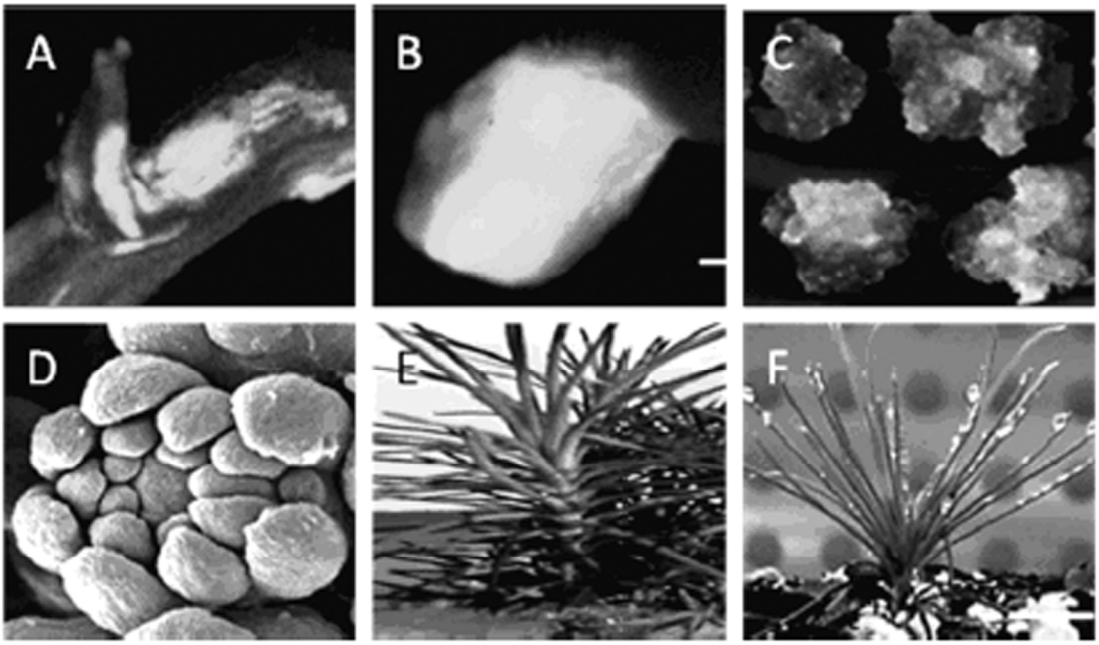

Fig. 4: The process of producing transgenic pine

Transgenic plants regenerated from kanamycin-resistant callus cultures in pine. A-C Kanamycin-resistant calli derived from cotyledons of mature zygotic embryos. D Scanning electronic microscopy of a shoot derived from transgenic callus on shoot formation medium for 5 weeks. E A cluster of shoots cultured on shoot formation medium for 6 weeks. F Twelve-week transgenic plantlet established in soil in the greenhouse .

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